Biotechnology Risk Assessment Research Grants Program
9706064 Risk Assessment of Release of Recombinant *Actinobacillus pleuropneumoniae*
T.J. Inzana, B. Fenwick, J. Longstreth, and H.P. Veit
Contact: Thomas Inzana
Department of Pathobiology
Center for Molecular Medicine & Infectious Diseases
Virginia Polytechnic Institute & State University
1410 Prices Ford Road
Blacksburg, VA 24061-0342
Phone: 540-231-4692
Email: tinzana@vt.edu

Specific Aims. Our overall objectives were to 1) determine the potential for transmission of a genetically modified strain (GMS) of the swine pathogen *Actinobacillus pleuropneumoniae* (Ap) from immunized pigs to nonimmunized, contact pigs; 2) determine the potential for transmission of any foreign DNA, particularly the kanamycin antibiotic resistance genes into other microorganisms. The original objectives were designed to be carried out in two phases. The objectives of the first phase were to: 1) Determine the optimum dose of virulent Ap that will induce a sublethal, but infectious and transmissible exposure to contact pigs; 2) Challenge pigs intranasally with a nonencapsulated GMS of Ap serotype 5 (J45-100) at the predetermined infectious dose. Transmission of the challenge strain to contact pigs, or the GMS's antibiotic resistance gene to other microorganisms will be determined; 3) Challenge pigs intramuscularly with J45-100 as above to simulate natural release into the environment. Transmission of the challenge strain to contact pigs, or the GMS's kanamycin resistance gene to other microorganisms, will be determined; 4) Challenge pigs intranasally with a virulent, GMS of Ap at the predetermined infectious dose to enhance infections and transmission. Transmission of the challenge strain to contact pigs, or the GMS's antibiotic resistance gene to other microorganisms, will be determined.

Results. The phase I work on this project has been completed. Using a spontaneous nalidixic acid resistant (Nal^R) mutant of encapsulated Ap in 3 separate studies we determined that approximately 10^6 bacterial cells of virulent Ap administered in the trachea (IT) or 10^9 bacterial cells of virulent Ap administered in the nose (IN) could establish a sublethal clinical or subclinical infection in exposed pigs. Ap was recovered from 3 of 6 contact pigs for as long as 7 days after contact with pigs challenged IN. Similar results were obtained with the IT group, in which Ap could be recovered from nasal swabs of 4 of 6 contact pigs at least 7 days after exposure. Once the transmissible dose of Ap was established groups of pigs were challenged with $2x10^9$ bacterial cells of nonencapsulated GMS J45-100 by the intramuscular (IM) and IN routes (Table 1). J45-100 was readily recovered from all IN challenged pigs up to 24 hours after challenge, but was not recovered from any contact pigs. J45-100 could only be recovered from the immunization site of the IM group, and not from the respiratory tract of the immunized pigs. As a result, the IM group was not tested for non-Ap kanamycin resistant bacteria. From the IN groups a total of 51 non-Ap colonies recovered from nasal swabs were kanamycin resistant. Colony hybridization blotting with a probe to the Kan^R gene identified 24 weakly reactive colonies. However, polymerase cahin reaction (PCR) using primers specific to

the Kan^R gene failed to amplify any product from these colonies, but did amplify the Kan^R gene from J45-100 recovered from challenged pigs.

In separate studies, pigs were challenged with an Ap 4074 serotype 1 mutant that has a single gene in its capsule biosynthesis region deleted (strain 4074-2365). This mutation resulted in a GMS that made a structurally altered capsule, resulting in a reduction but not complete loss of virulence. Groups of pigs were given an IT challenge (10^6-10^7 CFU), or an IN or IM challenge ($2x10^9$ CFU) of this strain. Ap was recovered for up to 48 hours from all IT and IN challenged pigs, but not from any of the contact pigs. All pigs challenged IM with strain 4074-2365 became seriously ill. One died between 24 and 48 hours and strain 4074-2365 was recovered from its lungs; the remaining pigs had to be euthanized between 3 and 10 days after challenge. From these groups of pigs 219 non-Ap, kanamycin resistant colonies were recovered from the respiratory tract and 39 were recovered from the GI tract (Table 1). Colony hybridization identified 33 kanamycin resistant colonies , but no product was amplified from these colonies by PCR, indicating the Kan^R gene was not present. The Kan^R gene was amplified from 15 colonies of 4074-2365 recovered from the challenged pigs. The Kan^R gene present in the chromosome of J45-100 and in 4074-2365 was not detected in any non-Ap bacteria recovered from any sites of either contacts or exposed pigs. Furthermore, although the GMS was recovered from exposed pigs up to 7 days following challenge, neither GMS was recovered from any of the contact pigs.

A phase II trial is ongoing in a herd in Colorado. About 30% of the pigs in this herd have been seriously affected by Ap pleuropneumonia. Swabs were obtained from the environment and from anterior portion of the noses of pigs. These swabs were collected to determine the frequency of kanamycin resistant bacteria in the environment. About 1200 pigs were then aerosol-inoculated

TABLE 1. Recovery and Transmission of Recombinant Ap and its Kan^R Gene to Contact Pigs in a Closed Environment.

Challenge strain	Challenge route	Number of pigs (E or C)[a]	# of Kan^R non-Ap colonies recovered	# of Kan^R Ap colonies recovered	# of non-Ap colonies with Kan^R gene recovered
J45-100[b]	IN	4 (E)	20	8	0
J45-100	IN	3 (C)	31	0	0
J45-100	IM	3 (E)	Not screened	0	0
J45-100	IM	3 (C)	Not screened	0	0
4074-2365[c]	IM	8 (E)	Not screened	0	0
4074-2365	IM	3 (C)	Not screened	0	0
4074-2365	IT	4 (E)[d](10)	67	0	0
4074-2365	IT	4 (C)[d](9)	68	0	0
4074-2365	IN	4 (E)[d](10)	37	15	0
4074-2365	IN	4 (C)[d](10)	47	0	0

[a]E = exposed pigs; C = contact pigs.

[b]J45-100 – a nonencapsulated, avirulent allelic exchange mutant containing a Kan^R gene.

[c]4074-2365 – a semi-virulent allelic exchange mutant with one mutated gene in capsule biosynthesis containing the Kan^R gene resulting in a structurally modified capsular polysaccharide.

[d]Fecal samples were also tested from these pigs to determine if the Kan^R gene may have moved into an enteric bacterium. As expected, Ap was not recovered from any fecal samples. Kanamycin resistant enteric bacteria were recovered (in parentheses), but all were negative for the Kan^R gene by colony hybridization and PCR.

with strain J45-100 using a hose-misting system that has been confirmed to inoculate pigs with vrulent bacteria, in which they become infected, clinically ill, and some die. None of the pigs exposed to J45-100 developed any clinical signs. Swabs of the environment and nasal swabs

were again collected about 10 days after exposure. The data obtained from culture of these swabs is currently being analyzed and tabulated. At this time we do know that a substantial number of bacteria in the pigs and the environment were kanamycin resistant before exposure to the organism. In swabs from the environment there was 0% to 50% less growth of bacteria on media containing kanamycin that without it. From nasal swabs there was 50% to 75% less growth on media containing kanamycin. After exposure of pigs to the GMO these percentages remained relatively unchanged. However, none of the kanamycin resistant colonies thus far screened have been shown to contain the Kan^R gene of Tn903. Furthermore, strain J45-100 was not recovered from any of the nasal or environmental swabs 10 days following exposure. All swabs from the culture used for misting did contain viable J45-100 containing the Kan^R gene. In summary, we found that the risk antibiotic gene transfer within the chromosome of a nonencapsulated strain of Ap to other bacteria, or transmisiion of this atenuated strain to other pigs was very low, and that such vaccines should be considered safe. Statistical and risk assessment analysis is currently being conducted to obtain a more exact calculation of the risk based on the number of bacterial colonies screened.

Plans for the coming year. Because all the data at this point is negative we can only say that the risk of transmission of the Kan^R gene to heterologous bacteria, or recombinant Ap to contact pigs is very low. Dr. Janice Longstreth and the statistical analysis laboratory at The Virginia-Maryland Regional College of Veterinary Medicine are currently doing a statistical analysis of the data using various programs from the SAS® system to obtain semi-quantitative risk assessment. However, Bayes' Theorem with a uniform (0,1) prior distribution and a binomial likelihood function will be applied to the data to calculate the potential rate of gene transfer based on the number of colonies screened.

Publications. Glindemann, G., J. Longstreth, B. Fenwick, and T.J. Inzana. 2000. Investigation of Transmission of Nonencapsulated, Recombinant *Actinobacillus pleuropneumoniae* or its Antibiotic Resistance Gene to Contact Pigs or Bacteria. Amer. Soc. Microbiol. Gen. Meet., May, 2000, Los Angeles, CA. Manuscript of this work in preparation.